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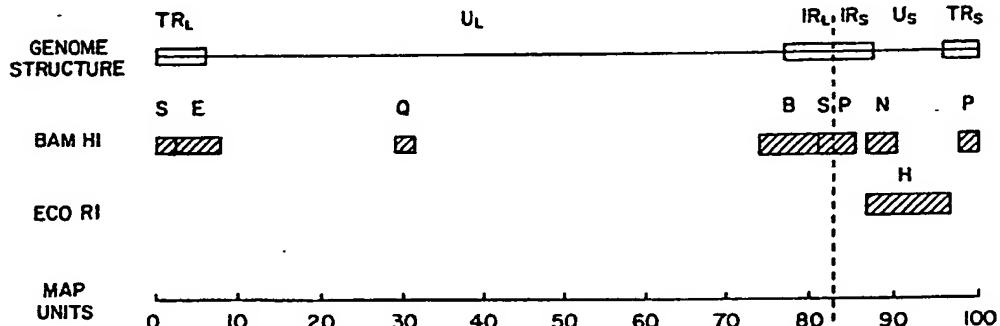
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ³ : C12Q 1/70		A1	(11) International Publication Number: WO 84/00979 (43) International Publication Date: 15 March 1984 (15.03.84)
<p>(21) International Application Number: PCT/US83/01209</p> <p>(22) International Filing Date: 8 August 1983 (08.08.83)</p> <p>(31) Priority Application Number: 411,015</p> <p>(32) Priority Date: 24 August 1982 (24.08.82)</p> <p>(33) Priority Country: US</p> <p>(71) Applicant: THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY [US/US]; 36th and Spruce Streets, Philadelphia, PA 19104 (US).</p> <p>(72) Inventors: FRASER, Nigel, W. ; 37 University Mews, 45th and Spruce Streets, Philadelphia, PA 19104 (US). ROCK, Daniel, L. ; 3545 Indian Queen Lane, Philadelphia, PA 19129 (US).</p> <p>(74) Agent: ROGERS, Gordon, S.; Howson and Howson, 13th Floor Three Parkway, Philadelphia, PA 19102 (US).</p>		<p>(81) Designated States: AT, AT (European patent), BE (European patent), CH, CH (European patent), DE, DE (European patent), FR (European patent), GB, GB (European patent), LU (European patent), NL (European patent), SE (European patent).</p> <p>Published <i>With international search report.</i></p>	

(54) Title: PROCESS FOR DETERMINING WHETHER A HERPESVIRUS IS IN THE VIRULENT OR LATENT STATE



(57) Abstract

A test for determining whether a herpesvirus present in cellular tissue is in the virulent or latent state which comprises extracting DNA from cells suspected of being infected with a herpesvirus, digesting the DNA with a restriction endonuclease, separating the resulting DNA fragments by size, transferring the separated DNA fragments to a nitrocellulose sheet, hybridizing the separated DNA fragments to radio active-labelled nick-translated terminal fragments of the herpes viral genome, subjecting said hybridized DNA fragments to autoradiography, and comparing the pattern of autoradiograph bands obtained with the pattern of autoradiograph bands produced by the hybridization of a restriction enzyme digest of DNA from cells infected with the same type of virulent herpesvirus to radio active-labelled nick-translated terminal fragments of the herpesvirus genome.

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Description

Process for Determining whether a
Herpesvirus Is in the Virulent or Latent State

Four types of human herpesviruses can be
5 identified: cytomegalovirus, Epstein-Barr virus,
varicella zoster virus, and herpes simplex virus types 1
and 2 (HSV-1 and HSV-2, respectively). Of these HSV-1
has been studied most and, almost from its discovery,
has been known by its ability to form latent infections
10 in nervous tissue (Goodpasture, E.W. (1929) Medicine
(Baltimore) 8, 223). The virus reactivates periodically
to produce recurrent disease (Stevens, J.G., (1978) Adv.
Cancer Res. 26, 227-257; Klein, R.J. (1982) Archives of
15 Virology 72, 143-168). HSV-1 has been found in the
human brain, as for example in the frontal lobe gray
matter, periventricular white matter, cerebellum and
pariental lobe, as well as the trigeminal ganglia.

The genome of HSV-1 is a linear
double-stranded DNA of 10^8 daltons (Kieff, E.D. et al.
20 J. Virol. 8, 125-132 (1971)) and consists of two unique
segments bounded by inverted repeats (Sheldrick, P. et
al (1974) Cold Spring Harbor Symp. Quant. Biol. 39,
667-678). The G + C content of the genome is unusually
high (67%; Kieff et al, (1971) J. Virol. 8, 125-132),
25 and it is cut by restriction endonuclease BamHI
(G↓G-A-T-C-C) 40 times.

There has been a need not only to detect the
presence of herpesvirus in cellular tissue, but also to
determine whether it is in the virulent, as compared to
30 the latent stage, in the absence of clinical signs of
the disease, such as cold sores, since the virus, when



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virulent, is some instances, can and does attack and destroy portions of the brain.

Objects of the Invention

5 A primary object of the present invention is to provide a novel process for determining whether a herpesvirus which is believed to have infected cellular tissue is in the virulent or latent state.

10 Another object of the invention is to provide a molecular test for the presence of virulent herpes simplex virus type 1.

These and other objects of this invention will become apparent from the following specification, appended claims, and drawings, in which:

15 Fig. 1, Lane 1, is an autoradiograph of blot filters showing patterns of bands as a result of digesting DNA from CV-1 cells infected with HSV-1 (F) with restriction endonuclease BamHI and hybridizing the digested DNA with ^{32}P -labeled nick-translated HSV-1 (F) total virion DNA probe; Lanes 2 to 13 bands from 20 similarly digested and hybridized DNA from mouse cell sources, and Lanes 14 and 15 represent similarly digested and hybridized DNA from uninfected mouse cell sources;

25 Fig. 2 is a map of the prototype HSV-1 genome, and

Fig. 3 is an autoradiograph generally similar to that of Fig. 1 using a ^{32}P -labeled nick-translated HSV-1 strain F (SP) fragment probe.

Details of the Invention

30 The process of this invention is designed to determine whether a herpesvirus is in the active or virulent state, as compared to the latent state, in



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cellular tissue believed to be infected with a herpesvirus. Although the process is described in detail with respect to determining whether HSV-1 is in the active or latent state, it is to be understood that 5 the process is equally applicable to other types of herpesviruses such as HSV-2, cytomegalovirus, Epstein-Barr virus and varicella zoster.

As noted above, HSV-1 is found in the brain and trigeminal ganglia and may be in the active form in 10 the absence of clinical signs of the infection. In the process of the invention, brain or other cellular tissue suspected of being infected with HSV-1 is obtained and DNA is extacted therefrom. The DNA so obtained is digested with a restriction endonuclease to cut the 15 genome into a number of fragments, these fragments are separated by size, and the separated fragments are bound to filter sheets and hybridized to radio active-labeled nick-translated terminal fragments of the HSV-1 genome. The resulting hybridized DNA fragments are then subjected to autoradiography, and the pattern 20 of bands obtained is compared with the pattern of autoradiograph bands produced by the hybridization of a restriction endonuclease digest of DNA from cells infected with HSV-1 to radio active-labeled 25 nick-translated terminal fragments of HSV-1.

The DNA may be extracted from the cells suspected of being infected with HSV-1 by means of the following procedure. Approximately 1 or 2 cm³ of tissue are minced in 0.15M NaCl/0.05M EDTA/0.01 M Tris-HCl, pH 30 8.0, at 4°C. This material is throughly homogenized, as for example in a Dounce homogenizer, brought to a final concentration of 0.5% in NaDODSO₄, and incubated



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overnight with self-digested Pronase at 500 μ g/ml. The DNA is extracted with phenol/chloroform and precipitated with 2 volumes of ethanol. The precipitate is centrifuged out and dissolved in 0.01 M Tris·HCl/0.01 M 5 EDTA, pH 7.4. Ribonuclease A is added to 40 mg/ml, the solution is incubated at 37°C for 2 hours, extracted with phenol, and the DNA is precipitated with ethanol. The DNA recovered is quantitated by measuring A_{260} (1 μ g/ml = 0.02 A unit).

10 The DNA so extracted is then digested with a restriction endonuclease, examples of which are BamHI, SmaI, and XmaI, all of which are available from New England Bio Labs. BamHI and SmaI are also available from Bethesda Research Laboratories, Inc. The preferred 15 restriction endonuclease is BamHI since although it cuts the terminal repeats in the HSV-1 genome (see Fig 2), it does not cut the viral genome into as many fragments as SmaI and XmaI, and thus make the process somewhat less complex.

20 In the digestion step 20 units of the restriction endonuclease, e.g. BamHI, may be used to digest 10 μ g of DNA in 20 μ l of 100 mM Tris·HCl, pH 8.0/7 mM MgCl₂. After incubation for 2 hours at 37°C an additional 20 units of enzyme are added and digestion is 25 continued for a further 2 hours to minimize the possibility of partial digestion products.

30 Separation of the fragments of digested DNA by size may be accomplished by electrophoresis on 0.5% agarose gels (Bio-Rad) in a borate buffer (0.09 M Tris·HCl, pH 8.3/0.9 M boric acid/0.25 M EDTA containing ethidium bromide at 0.05 μ g/ml). The gels are then blotted onto nitrocellulose filters.



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For further details of the steps of restriction endonuclease digestion, electrophoresis and transfer or blotting into nitrocellulose sheets, see Southern, E.M. (1975) J. Mol. Biol. 98, 503-517 and 5 Wahl, G.M. et al, (1979) Proc. Natl. Acad. Sci. USA 76, 3683-3687.

As noted above, after transfer of the separated DNA fragments onto the nitrocellulose sheets or filters, the fragments are hybridized to radio active-labeled nick-translated terminal fragments of the same herpes viral genome as is suspected of infecting the test cells. These radio active fragments may be cloned using the procedure described by Roizman, B., (1979) Cell 16, 481-494.

10 In order to determine the presence of the virus, nick-translated probes of the total virion may be prepared by the techniques of Maniatis, T. et al (1976) Cell 8, 163-182, and Rigby , P.W. J., et al (1977) J. Mol. Biol. 113, 237. In such procedure, to 0.25 μ g of 15 HSV-1 DNA purified from virions in 25 μ l of 50mM Tris·HCl/5mM Mg Cl₂/bovine serum albumen (50 μ g/ml)/1mM dGPT/1mM dTTP are added 100 Ci (1 Ci=3.7x10¹⁰ becquerels) of [α -32P]dCTP (Amersham; 200-300 Ci/mM), 20 2x10⁻⁴ Kunitz units of DNase 1 (Worthington), and 2 25 units of DNA polymerase purified from E. Coli (Boehringer Mannheim). The reaction mixture is incubated at 15°C for 2 hours, and then 100 μ l of 0.1 M NaCl/10mM Tris·HCl, pH 7.4/1mM EDTA/0.1% NaDODSO₄ containing 50 μ g of sonicated salmon sperm DNA is added.

30 The solution is passed through a Sephadex G-50 column, and the peak fractions of DNA are ethanol precipitated, specific activities of 3-7 x 10⁸ cpm/g ordinarily may be achieved with a 30-50% incorporation of label.



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Preferred radio activ probes for use in the process of the present invention are cloned ^{32}P -labeled nick-translated (SP) terminal fragments of the HSV-1 viral genome prepared by the method of Roizman B.,
5 supra.

Following transfer of DNA fragments onto nitrocellulose filters, the transferred fragments, referred to in the art as "blots", are hybridized to the radio active nick-translated terminal fragment probes of
10 the type described hereinabove.

The "blot" filters are maintained in 10 ml of 20% formamide/0.6M NaCl/0.06 M sodium citrate/0.01 M EDTA/0.1% NaDODSO₄/5-fold Denhardt's solution (see Denhardt, D. (1966) Biochem. Biophys. Res. Commun. 23,
15 641-646) containing sonicated denatured salmon sperm DNA at 50 $\mu\text{g}/\text{ml}$ in 1/2 gal plastic bottles at 50°C on a standard tissue culture roller bottles apparatus for 5-16 hours prior to addition of denatured radio active probe (1-5x10⁷ cpm per filter).

20 Hybridization is carried out during a period of 36 hours at 50°C in 50% formamide/10% dextran sulfate (see Wohl et al supra)/Denhardt's solution/4-fold standard saline citrate (NaCl/Cit/0.1M EDTA/0.1% NaDODSO₄ containing sonicated denatured salmon sperm DNA
25 at 25 $\mu\text{g}/\text{ml}$.

To remove unhybridized labeled material, the nitrocellulose filters are rinsed twice with 5-fold NaCl/Cit/0.1% NaDODSO₄ at 50°C for 10 minutes each and then for 20 minutes with hybridized mixture without
30 salmon sperm DNA and dextran sulfate at 50°C. The filters are rinsed once in NaCl/Cit/0.1% NaDODSO₄ at 37°C for 20 minutes, once in NaCl/Cit/0.1% NaDODSO₄ at



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65°C for 20 minutes, once in half-strength NaCl/Cit/0.1% NaDODSO₄ at 65°C for 20 minutes and finally in quarter-strength NaCl/Cit/0.1% NaDODSO₄ at 65°C for 15 minutes.

5 The filters are blotted dry and autoradiographed with XR film and DuPont Cronex Lighting Plus screens at -70°C for 1-10 days.

For purposes of comparison a control hybridized restriction enzyme digest of DNA from cells 10 infected with the same type of virulent herpesvirus as that believed to infect the test cells to radio active-labeled nick-translated terminal fragments of the same herpesvirus genome is prepared using the above described procedures, and subjected to autoradiography. 15 The respective autoradiographs of the test specimens are compared to those of the latter known control specimens and matching bands representing terminal fragments of the viral genome indicate the presence of virulent herpesvirus in the test specimen. Whether the test 20 cells are infected with a herpes virus will also be indicated by the correlation of other bands of the respective specimens, the absence of bands representing the terminal fragments indicating that the virus is in the latent state.

25 The invention will be further described in connection with the following specific examples which are given by way of explanation and are not intended to in any way limit the scope of the invention.

Example I

30 The purpose of this example is to determine the presence of HSV-1 in the cellular tissue of the test animals.



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BALB/c mice were infected with HSV-1(F) by corneal scarification and DNA was extracted from mouse brain and ganglia at acute and latent stages of infection (see Puga, A et al, (1979) *Virology* 89, 102-111). DNA was extracted from ganglia and brains of latently infected mice, digested with restriction endonuclease BamHI, the DNA fragments were separated by agarose gel electrophoresis, the separated fragments were transferred to nitrocellulose filters and hybridized to cloned ^{32}P -labeled, nick-translated fragments of the HSV-1 total viral genome using the procedure which has been detailed hereinabove. The control was similarly prepared using DNA from the HSV-1 (F) strain which was grown on CV-1 and baby hamster kidney (BHK)-21 cells. Autoradiographic exposures ranged from 1 to 6 days.

Fig. 1 shows a selection of acutely infected and latently infected mouse brain and ganglia DNA. Lane 1, the control, represents 1.0 ng. of HSV-1 (F) DNA, purified from extracellular virions and $10\mu\text{g}$ of uninfected CV-1 cell DNA. Lanes 2 to 6 represent $20\mu\text{g}$ of mouse brain DNA taken from five acutely infected mice (6 days post inoculation). Lanes 7, 8 and 10 to 13 represent $20\mu\text{g}$ of brain DNA taken from six latently infected mice (2 months post inoculation). Lane 9 represents $20\mu\text{g}$ of DNA extracted from pooled latently infected trigeminal ganglia (2 mo. PI). Lanes 14 and 15 represent $20\mu\text{g}$ of brain DNA taken from each of two uninfected control animals.

Using the above technique where total viron DNA was used as the probe (see Maniatis et al and Rigby et al, *supra*), the presence of HSV-1 (F) was detected in the test animals shown in Table I, below:



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TABLE I
 DETECTION OF THE HSV-1 GENOME IN
 MOUSE BRAIN BY BLOT HYBRIDIZATION

A. WHOLE BRAIN

5	Time Post Infection	Positive Samples	/	Total No. of Samples	Genome Equivalents Per Cell		
					1.5	-	0.15
	6 days	5/5					
	2 mo.	7/15			0.15	-	0.015
	5 mo.	8/15			0.15	-	0.015

10

B. BRAIN REGIONS

	Mouse No.	Brain Stem (Pons-Medulla)	Cerebellum	Cerebrum
15	068	+	-	+
	069	+	-	+
	070	+	-	-
	071	++	-	-
	072	++	-	-

20 DNA was extracted from whole brain at various times PI or from brain regions at 2 mo. PI twenty μ g samples of brain DNA were digested with BamHI, electrophoresed in agarose gels, transferred to nitrocellulose filters, and hybridized with ^{32}P labeled HSV-1 strains (F) DNA. Genome equivalents was approximated by visual comparison of the sample autoradiographic image with that of reconstructed standards.

25 (+) denotes a sample positive for HSV-1 DNA with 0.15-0.015 genome equivalents per cell, (++) indicates



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the sample contained about 0.15 but less than 1.5 genome equivalents per cell. (-) denotes samples that were negative for HSV-1 DNA under the assay conditions.

As can be seen from Table I, Part A, between 5 and 5 months post inoculation (PI), approximately half the mouse brains examined were positive. However, when anatomically defined regions of the brain were examined all of the latent infected mice were positive for HSV-1 (see Part B of Table I). The stem which constitutes 10 about 1/15 (50 μ g) of the brain DNA appears to be rich in latent viral DNA which is to be expected if the method of brain infection by HSV-1 is via the trigeminal nerve route.

Referring to Fig. 2, the prototype HSV-1 (F) genome there shown consists of a long unique region (U_L) and short unique regions (U_S). Both unique regions are bounded by inverted repeat regions (TRL , IR_L , IR_S , TR_S). The long and short segments of the genome invert relative to one another forming four isomers. The 20 fragment BamHI SP which spans the junction of the long and short segments of the genome is totally within the IR_L and IR_S regions resulting in a single BamHI junction fragment for all isomers. Cloned viral fragments used as probes in blot hybridization experiments are 25 localized along the genome map. The genome illustrated in Fig. 2 is divided into 100 map units, each of which represents 0.98×10^6 daltons or approximately 1.63 kilobases.

EXAMPLE 2

30 Rather than using total virion DNA as the probe for viral sequences as in Example 1, the probe used was the BamHI fragment SP corresponding to the termini and joint regions (see Fig. 2), which was hybridized with virion DNA (control) and mice brain DNA.



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Fig. 3 shows a blot hybridization using BamHI (SP) fragment DNA as the probe. By comparison of Lane 1 (viron DNA control) and Lanes 2 to 4 (acute brain DNA), it can be seen that the bands containing the termini of the genome are sub molar in amount. This is not due to isomerization of the viral genome because BamHI cuts with the terminal repeat and therefore each isomer gives the same terminal fragment (see Fig. 2). Lanes 5 to 9 show brains infected wth latent virus and no terminal fragments can be seen. Densitometer scans for the virion, acute and latent autoradiographs showed a molar ratio relative to the PS joint fragment of 0.3 for the P and S terminal fragments during acute infection. That the terminal fragments are reduced in molarity during acute infection of neural tissue with HSV-1 is not unusual. It has previously been noted in infected tissue cells (see Jacob, R.J. et al. (1979) *Virology* 29, 448-457). However, that no terminal fragments appear to be present in brain or ganglia infected with latent virus was most surprising, and clearly indicates that the viral DNA which enters the latently infected cells is modified during initial phase of latency and does not merely reside inside the cell. As neuronal cells do not undergo DNA replication, it is believed that the viral DNA is not integrated as a unit length molecule into cellular DNA. More probably the viral DNA detected in these latently infected cells represents concatemers of viral DNA which are seen typically as intermediates in rolling circle models of DNA replication.

The scientific papers referred to in this specification are incorporated herein in thier entirety by reference.



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The research work herein described was supported by grant from the National Institute of Neurological and Communicative Disorders and Stroke.



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Claims

1. A test for determining whether a herpesvirus present in cellular tissue is in the virulent or latent state which comprises extracting DNA from cells suspected of being infected with a herpesvirus, digesting said DNA with a restriction endonuclease, separating the resulting DNA fragments by size, transferring said separated DNA fragments to a filter sheet, hybridizing the separated DNA fragments with radio active-labeled nick-translated terminal fragments of the herpes viral genome, subjecting said hybridized DNA fragments to autoradiography, and comparing the pattern of autoradiograph bands obtained, with the pattern of autoradiograph bands produced by the hybridization of a restriction enzyme digest of DNA from cells infected with the same type of virulent herpesvirus with radio active-labeled nick-translated terminal fragments of the herpesvirus genome.

2. The process according to claim 1 in which said herpesvirus comprises herpes simplex virus type 1.

3. The process according to claim 1 in which said radio active-labeled nick-translated terminal fragments of herpesvirus DNA comprise ^{32}P -labeled DNA fragments.

4. The process according to claim 1 in which said DNA fragments resulting from restriction endonuclease digestion are separated by gel electrophoresis, and the resulting pattern of DNA



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fragments is transferred to a nitrocellulose sheet for hybridization with said radio active-labeled nick-translated fragments of the herpes viral genome.

5. The process according to claim 1 in which said endonuclease comprises BamHI.

6. A test for determining whether herpes simplex virus type 1(HSV-1) present in a cellular tissue is in the virulent or latent state which comprises extracting DNA from cells suspected of being infected with HSV-1, digesting said DNA with the endonuclease BamHI, separating the resulting DNA fragments by gel electrophoresis, transferring the separated DNA fragments to a nitrocellulose sheet, hybridizing the separated DNA fragments with ^{32}P -labeled nick-translated terminal fragments of the HSV-1 viral genome, subjecting said hybridized DNA fragments to autoradiography, and comparing the pattern of autoradiograph bands obtained with the pattern of autoradiograph bands produced by the hybridization of a restriction enzyme digest of DNA from cells infected with virulent HSV-1 with ^{32}P -labeled nick-translated terminal fragments of the HSV-1 viral genome.



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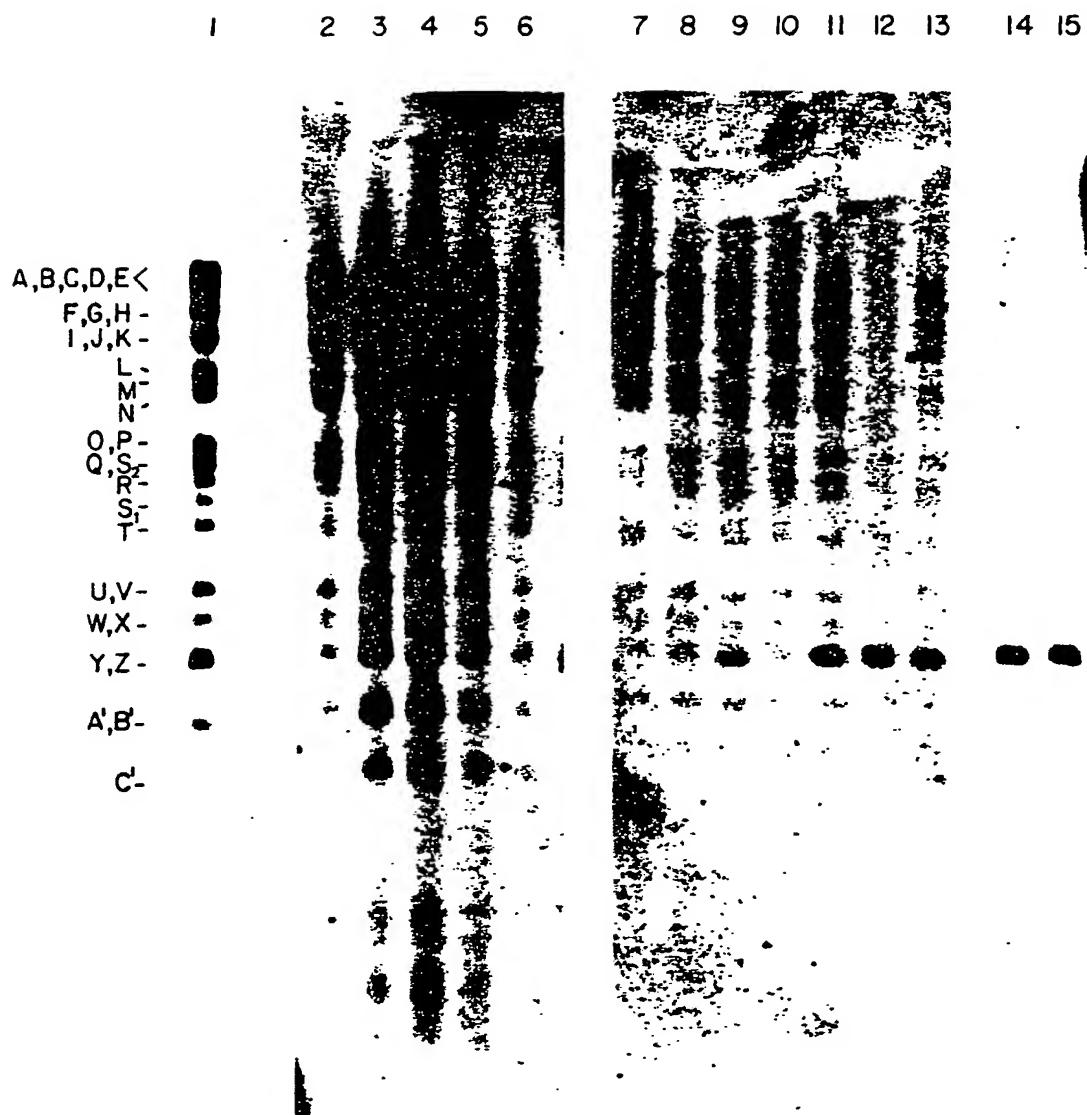


FIG. 1

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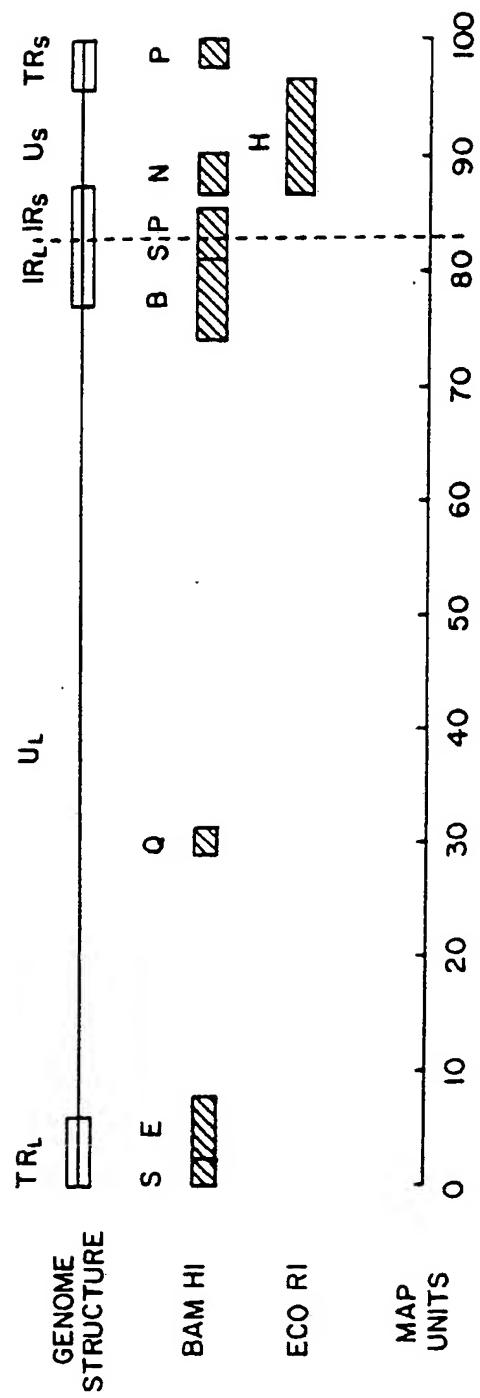


FIG 2



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1 2 3 4 5 6 7 8

JOINT
FRAGMENT (PS)

TERMINAL FRAGMENTS

SHORT SEGMENT (P)

LONG SEGMENT (S)



FIG. 3



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US83/01209

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. Cl. 435/5
INT. Cl. C12Q 1/70

II. FIELDS SEARCHED

Minimum Documentation Searched *

Classification System	Classification Symbols
U.S. 435/5, 6, 91, 172, 820, 948 436/804, 57	

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

CHEMICAL ABSTRACTS 1972-DATE HERPESVIRUS AND HYBRIDIZATION INDEX MEDICUS 1972-DATE, HERPESVIRUS AND HYBRIDIZATION

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
A, P	U.S. 4,358,535, 9 NOVEMBER 1982, FALKOW ET AL	1-6
A	U.S. 4,302,204, 24 NOVEMBER 1981, WAHL ET AL	1-6
X, A	N, JOURNAL OF VIROLOGY 38(2), MAY 1981, HELLER ET AL, "EPSTEIN-BARR VIRUS DNA IX. VARIATION AMONG VIRAL DNAs FROM PRODUCER AND NONPRODUCER INFECTED CELLS" p. 632-648	1-6
A	N, JOURNAL OF VIROLOGY 41(2), FEBRUARY 1982 GALLOWAY ET AL, "LIMITED TRANSCRIPTION OF THE HERPES SIMPLEX VIRUS GENOME WHEN LATENT IN HUMAN SENSORY GANGLIA" p. 686-691	1-6
A	N, PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, 78(10), OCTOBER 1981, FRASER ET AL, "HERPES SIMPLEX TYPE 1 DNA IN HUMAN BRAIN TISSUE" p. 6461-6465	1-6
A	N, JOURNAL OF VIROLOGY, 43(1), JULY 1982, HUMMEL ET AL, "EPSTEIN-BARR VIRUS RNA VIII. VIRAL RNA IN PERMISSIVELY INFECTED 395-8 CELLS" p. 262-272	1-6

* Special categories of cited documents: ¹⁵

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ¹

3 NOVEMBER 1983

Date of Mailing of this International Search Report ²

07 NOV 1983

International Searching Authority ¹

ISA/US

Signature of Authorized Officer ²⁰

JOHN E. TARCZA

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	N, PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, 79(6), JUNE 1982, MCDUGALL ET AL, "HERPESVIRUS-SPECIFIC RNA AND PROTEIN IN CARCINOMA OF THE UTERINE CERVIX" p. 3853-7	1-6
A	N, NATURE, 288, 20 NOVEMBER 1980, CABRERA ET AL, "HERPES SIMPLEX VIRUS DNA SEQUENCES IN THE CNS OF LATENTLY INFECTED MICE" p. 288-290	1-6

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.